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Europäisches Patentamt
European Patent Office
Office européen des brevets

(11) Publication number:

0070686
A2

(12)

EUROPEAN PATENT APPLICATION

(21) Application number: 82303700.7

(51) Int. Cl.: **G 01 N 33/58**

(22) Date of filing: **14.07.82**

(30) Priority: **17.07.81 US 284400**

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(43) Date of publication of application: **26.01.83**
Bulletin 83/4

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(84) Designated Contracting States: **BE CH DE FR GB IT LI NL**

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(54) **Non-radiative energy transfer immunochemical technique.**

(57) Antigen assays are performed by introducing an antigen-containing sample into a reagent solution comprising an absorber-emitter conjugated antibody and a chemiluminescent catalyst conjugated antibody, wherein the conjugated antibodies bind to the antigen in close proximity to enable transfer of energy to the absorber-emitter. The resulting emission of light from the absorber-emitter is related to the amount of antigen present in the sample.

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NON-RADIATIVE ENERGY TRANSFER IMMUNOCHEMICAL TECHNIQUEBACKGROUND OF THE INVENTION

Immunochemical type assays are rapidly becoming a major segment of the clinical diagnostic market.

5 Presently, nephelometric and radio-immunoassays (RIA) represent the most well established techniques in immuno-diagnostics. New techniques such as the enzyme multiplied immunoassay technique (EMIT), enzyme immuno-assay (EIA), and heterogeneous fluorescent immunoassays
10 have been introduced and have met with varying degrees of success. Most of these assay techniques are aimed at replacing RIA's because of the numerous problems involved in handling radioisotopes.

For example, U.S. 4,104,029 to Maier, Jr. teaches
15 a chemiluminescent assay method wherein a competition reaction is set up between chemiluminescent labeled antigens and sample antigens. These two species compete for the limited binding sites available provided by the antibodies present in the assay. The
20 resulting luminescence is inversely related to the amount of antigen in the sample.

Other methods have used the concept of non-radiative energy transfer, which occurs when a light emitting species is in close proximity to a light
25 absorbing species whose absorbance spectrum overlaps the emission spectrum of the emitting species. Under favorable conditions, all of the light energy which would otherwise be released by the emitter in the form of light is instead transferred to the absorbing species. This condition is described by the Forster equations (see "Energy Transfer and Organic Photo-chemistry", Technique of Organic Chemistry, Vol. XIV eds. P. A. Leermakers and A. Weissberger, pp. 17-132
30 (1969), Interscience, New York) which suggest that the rate of energy transfer is inversely proportional to
35 the sixth power of the distance between the absorbing

and emitting species. Hence it is necessary that the emitting and absorbing species be very close together to effect energy transfer.

For example, U.S. 3,996,345 to Ullman et al.

- 5 teaches a method for carrying out immunoassays employing antibodies and a fluorescer-quencher pair. Upon irradiation, the resulting fluorescense is related to the amount of the component of interest present. A number of U.S. patents by Ullman or Ullman et al. have
10 issued which are closely related in subject matter include 3,998,943; 4,160,016; 4,174,384; 4,161,515; and 4,199,559. All of these patents relate to the concept of fluorescent quenching in some manner.
Similar methods involving fluorescent quenching are
15 described in two articles. (See "Fluorescent Excitation Transfer Immunoassay," The Journal of Biological Chemistry, Vol. 251, No. 14, pp. 4172-4178 (July 25, 1976) and "Fluorescamine and Fluorescein as Labels in Energy-transfer Immunoassay," Analyst, Vol. 105, pp. 91-92 (1980).)
20 It has now been discovered that the specificity of antibody-antigen reactions can provide a means for bringing a chemiluminescent species and a light absorbing and re-emitting species in sufficiently close proximity to satisfy the energy transfer requirements.
25 This eliminates the need for an external source of exciting light and can result in a higher degree of sensitivity.

SUMMARY OF THE INVENTION

In one aspect, the invention resides in a method
30 for assaying antigens having multiple binding sites for antibodies comprising: (a) introducing an antigen-containing sample into a reagent solution comprising effective amounts of an absorber/emitter conjugated antibody, a chemiluminescent catalyst conjugated
35 antibody, and chemiluminescent reagents suitable for inducing a luminescent light response in the presence

of said chemiluminescent catalyst conjugated antibody; and (b) detecting the light response from the absorber/emitter conjugated antibody induced by the chemiluminescent light response. For purposes herein, the
5 term "absorber/emitter" refers to any species which absorbs light energy and emits light of a different wavelength. This term particularly includes fluorophores and phosphores. Also, the terms "chemiluminescent" or "chemiluminescence" include the closely related
10 phenomenon of bioluminescence. The term "antibody" shall include antibody analogs.

Because of the close proximity of the chemiluminescent catalyst conjugated antibody and the absorber/emitter conjugated antibody when bound to a
15 single antigen, a highly efficient energy transfer occurs from the chemiluminescent catalyst to the absorber/emitter. The absorber/emitter dissipates the energy by emitting light of a longer wavelength than the chemiluminescent light absorbed. Hence, in the
20 case of a fluorophore absorber/emitter, fluorescent light would only be substantially emitted when antigen was present in the sample. Any fluorescent light detected from the system would be related to the amount of antigen in the sample when light-labeled
25 reagent antibodies were present in nonlimiting amounts. The amount of fluorescent light emitted from the reagent solution as background light would be negligible because it is necessary that all of the appropriate reagents concurrently be very close together. In
30 solution the average distance between the chemiluminescent catalyst and the fluorophore would be such that negligible energy transfer would occur without the presence of antigen, which provides the binding sites (surface antigens) for the antibody conjugated reagents.
35 These binding sites are sufficiently close together to permit adequate energy transfer.

More specifically, it is a preferable aspect of this invention to include among the chemiluminescent reagents a "chemiluminescent cofactor generator catalyst" conjugated antibody which catalyzes the formation of a chemiluminescent cofactor. For purposes of this specification, a "cofactor" is any reactant species necessary to produce a light-emitting reaction, excluding the catalysts discussed herein.

Since both catalysts have been conjugated to an antibody which binds them to the antigen in close proximity, the likelihood of a chemiluminescent light response sufficient to excite the absorber/emitter is increased by the local generation of a necessary cofactor in the light-producing reaction.

It is also preferable to include among the chemiluminescent reagents a "chemiluminescent cofactor scavenger" catalyst. This catalyst remains free in solution and is not conjugated to an antibody. It serves to eliminate any particular cofactor which may be present in the reagent solution generally, since it is desirable to only have the necessary cofactors and other reagents present in the vicinity of the antigen-bound reagents. Hence, the presence of such a cofactor scavenger catalyst aids in reducing background light emissions by eliminating a key species in the light-producing reaction. On the other hand, the presence of the scavenger catalyst in the solution generally will not affect the assay since the concentration of the cofactor near the antigen is too high for the cofactor scavenger catalyst to have an appreciable effect. Hence, the desired positive reaction still occurs.

In a further aspect, the invention resides in a method for assaying antigens having only one binding site for antibodies comprising: (a) introducing a sample into a reagent solution comprising effective amounts of an absorber/emitter conjugated antigen, a chemiluminescent catalyst conjugated antibody, and

chemiluminescent reagents suitable for inducing a chemiluminescent light response in the presence of said chemiluminescent catalyst conjugated antibody; and (b) detecting the light response from the absorber-
5 emitter conjugated antigen induced by the chemiluminescent light response. (It is also suitable to tag the absorber/emitter onto the antibody and label the antigen with the chemiluminescent catalyst.) This aspect differs from the previous aspects in that the
10 reagent solution contains a light-labeled antigen, causing a competition reaction between the light-labeled antigen and the antigen in the sample for the light-labeled antibody. Hence, there is a certain amount of secondary light emitted in the absence of
15 sample antigen and a lesser amount of secondary light emitted in the presence of sample antigen.

In a further aspect, the invention resides in the reagent solutions described herein which are useful in carrying out the methods disclosed.

20

BRIEF DESCRIPTION OF THE DRAWING

Figure 1 illustrates one example of this invention using a specific reagent system in the presence of sample antigen.

25

Figure 2 illustrates the same system as in Figure 1,

but without the presence of sample antigen.

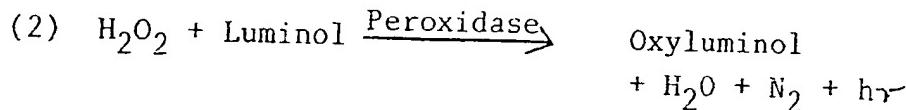
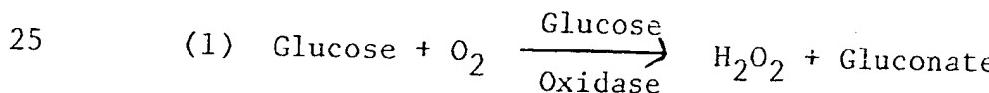
DETAILED DISCUSSION OF THE INVENTION

30 35

In carrying out the methods of this invention, the highly specific antibody-antigen reactions are used to bring together, in very close proximity, an excitable fluorescent or phosphorescent light source and an exciting chemiluminescent light source. The purpose of utilizing this principle is to achieve a highly sensitive and reliable method for assaying antigens in a given sample. The antigen-antibody affinity provides a means for accomplishing this

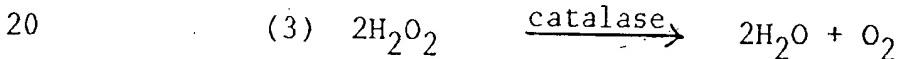
purpose by affording a unique way of bringing together certain necessary reactants which emit a characteristic light response when sample antigen is present.

- 5 Directing attention to Figure 1, the invention can be more easily understood by examining a specific representative example. Figure 1 illustrates the various reactions which take place when an antigen is present in the reagent solution as a result of combining the reagent solution with the sample to be assayed.
- 10 10 The antigen contains a plurality of binding sites which provide places for the various reagents to attach themselves. Shown are a fluorophore conjugated antibody (absorber/emitter conjugated antibody), a peroxidase conjugated antibody (chemiluminescent catalyst conjugated antibody), and a glucose oxidase conjugated antibody (chemiluminescent cofactor generator catalyst conjugated antibody), all necessarily bound and concentrated in a small space relative to the balance of the reagent solution. This close proximity of reactant species creates a much greater likelihood of a fluorescent light-emitting reaction. The pertinent chemiluminescent reactions for this particular reagent system are as follows:
- 15 20



- 30 As can be seen from reaction (2), both hydrogen peroxide (H₂O₂) and luminol must contact the peroxidase catalyst in order for a light-emitting reaction to occur. To this end it is possible (and within the scope of this invention) to simply have sufficient amounts of both cofactors (hydrogen peroxide and luminol) in the reagent solution, but such a situation
- 35

would lead to considerable background chemiluminescent light being emitted regardless of whether or not antigen is present. This background light could be filtered, but such a situation is not preferable. To 5 avoid an undue amount of background emissions, a chemiluminescent cofactor generator catalyst conjugated antibody (a glucose oxidase conjugated antibody) is included in the reagent solution. This goes one step further in assuring that chemiluminescence 10 will most likely occur only if antigen is present, since one of the necessary cofactors (H_2O_2) is only produced in the vicinity of the antigen-bound peroxidase catalyst. As further insurance against unwanted, chance chemiluminescent emissions occurring, the 15 reagent solution illustrated in Figure 1 contains a chemiluminescent cofactor scavenger catalyst (catalase) which removes hydrogen peroxide from the reagent solution generally according to the following reaction:

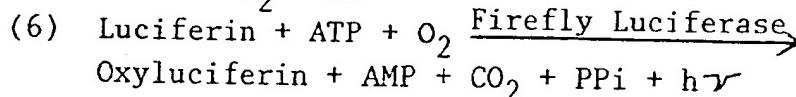
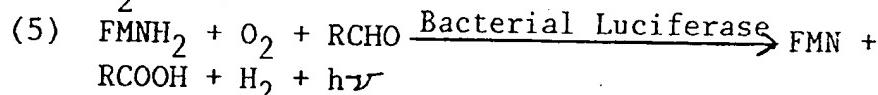
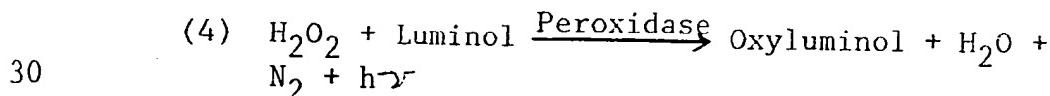


Hence, Figure 1 illustrates how glucose oxidase, peroxidase, and a fluorophore are all closely held in the vicinity of each other by being bound to the antigen with conjugated antibodies. Glucose and 25 oxygen in the reagent solution contact the glucose oxidase and form hydrogen peroxide. The hydrogen peroxide, being generated close to the peroxidase catalyst, combines with luminol present in the reagent solution to emit chemiluminescent light energy ($h\nu \geq 500 \text{ nm}$). However, because of the close proximity of 30 the peroxidase and the fluorophore, which is also closely bound to the antigen, this emitted light energy is absorbed and converted to fluorescent light by the fluorophore ($h\nu \leq 500 \text{ nm}$). Any chemiluminescent light is filtered, allowing the detector only 35 to detect the fluorescent light, which is proportional to the amount of antigen present in the sample.

Figure 2 illustrates the same reagent system of Figure 1, but without antigen present in the sample. Because all the reagents necessary to produce the fluorescent light response are randomly dispersed in solution, the likelihood of chemiluminescence occurring is small, particularly so because of the presence of catalase to scavenge any hydrogen peroxide formed. The chances of a secondary light emission (fluorescence) occurring is even smaller, since it is necessary that the excited chemiluminescent emitter be formed very close to the fluorophore. Otherwise there will be insufficient energy transfer to accomplish a detectable fluorescent response.

The necessary antibodies to be used for preparing reagents can be obtained by known methods, such as isolation of antibodies from the blood of an animal previously injected with the antigen of interest (See Herman N. Eisen, Immunology, Ch. 15 and 17, Harper Row, New York, 1974) or fusing an antibody producing cell with a myeloma cell to produce a stable producer of monoclonal antibodies (See R. H. Kennet and T. J. McKearn (eds.) Monoclonal Antibodies, Plenum Press, New York, 1981). Both references are hereby incorporated by reference.

Suitable chemiluminescent catalysts include peroxidase, bacterial luciferase, and firefly luciferase, which produce light according to the following separate reactions:



wherein $FMNH_2$ is reduced flavin mononucleotide, R is straight carbon chain having about 8-12 carbons, FMN is flavin mononucleotide, ATP is adenosine triphosphate,

AMP is adenosine monophosphate, and PPi is inorganic phosphates.

In choosing the particular absorber/emitter for a given reagent system, it is necessary that it possess absorbance in the spectral region of the chemiluminescence produced by the chemiluminescent reagents. It is preferable that the emission of the absorber/emitter be of a long enough wavelength to be effectively distinguished from the chemiluminescence emitted by the reagent system. For example, two chemiluminescent reactions of primary interest are luminol oxidation by hydrogen peroxide and aldehyde oxygenation (e.g. isobutyraldehyde and propanal). Both of these reactions are catalyzed by peroxidase. Suitable absorber/emitters for the luminol chemiluminescent reaction include free base porphyrins such as uroporphyrin and tetracarboxyphenylporphyrin, metalloporphyrins containing such metals as magnesium or zinc, tetraphenylcarboxyporphyrins, perylene, anthracene, 7-methyldibenzo (a,h) pyrene, and other polycyclic aromatics having conjugated ring systems of sufficient size to produce strong absorbance in the region of luminol chemiluminescence (between 400 and 450 nm). The absorber/emitters may be easily sulfonated and activated for conjugation with antibodies by formation of the sulfonic acid chlorides by general synthetic procedures. Also, carboxylation may be performed if required and acid chlorides formed of these to activate for coupling to antibodies.

Suitable absorber/emitters for the chemiluminescence resulting from aldehyde oxygenation include the above-mentioned porphyrins and polynuclear aromatics. However, halogenation of the polynuclear aromatics is required in order to provide efficient transfer of energy from the chemiluminescent emitter since it emits from a triplet excited state. Examples of appropriate halogenated polynuclear aromatics are 9,10-dibromoanthracene, 9,10-dibromo-2,6-anthracene

disulfonic acid, 3,10-dibromo-4,9-perylenedicarboxylate, and 3,9- or 3,10-dibromoperylene. If required, sulfonation or carboxylation as described are also easily performed on these compounds by general synthetic procedures.

5 procedures.

Phosphorescent compounds may also be used as absorber/emitters in the chemiluminescent aldehyde system due to the triplet nature of the emitter. In general, however, phosphorescent absorber/emitters are not preferable due to their sensitivity toward quenching in the presence of oxygen. This class of compounds includes α -diketones such as 2,3-butadione, 1[carboxy-phenyl]-1,2-pentanedione, and 1-phenyl-1,2-propanedione. In the latter case coupling to antibody or antigen is afforded through substitution of the phenyl ring with sulfonic acid chlorides, acid chlorides, or diazo activated intermediates.

The methods of attaching the absorber/emitters, chemiluminescent catalysts, and chemiluminescent cofactor generator catalysts to the appropriate antibodies will depend upon the particular species concerned. For example, the sulfonic acid derivative of a fluorophore such as 9,10-dibromo-2-anthracene sulfonic acid may be converted to the sulfonic acid chloride by treatment with POCl_3 in dichloroethane. The sulfonic acid chloride will then couple to the amino groups of lysine residues of antibodies or N-terminal amino acids in aqueous solution. The carboxylic acid derivative of a fluorophore such as dibromoperylene dicarboxylic acid may be converted to the acid chloride by treatment with thionyl chloride. The acid chloride will couple to antibody in the same manner as the sulfonic acid chlorides. These and other common coupling procedures are known in the literature (e.g. see Immobilized Enzymes, Antigens, Antibodies, and Peptides, H. H. Weetall (ed.), Ch. 1, Mareel Dekker, New York (1975) herein incorporated by reference). Many

peroxidase conjugates of antibodies are commercially available and are prepared by oxidation of carbohydrate residues on peroxidase to form an aldehyde through which amino groups of antibodies are reacted 5 forming Schiff base linkages. These Schiff bases are further stabilized by reduction with borohydride. Conjugation of glucose oxidase with an antibody has been reported using the bifunctional reagent succinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate 10 (S. Yoshitake *et al.*, European Journal of Biochemistry, Vol. 101, 395-399, herein incorporated by reference).

It will be appreciated that the chemiluminescent reagents or cofactors necessary for inducing a light response in the presence of the selected chemiluminescent catalyst conjugated antibody are well documented 15 and can easily be determined by those skilled in biochemistry. For example, in the presence of a peroxidase chemiluminescent catalyst, the light-inducing reagents or cofactors could include either (1) luminol and 20 hydrogen peroxide or (2) an aldehyde (such as isobutyraldehyde or propionaldehyde), dissolved oxygen, and ethanol and/or sodium pyrophosphate and/or sodium phosphate. In the presence of a firefly luciferase catalyst, the light-inducing reagents would include 25 the combination of luciferin, ATP, Mg⁺⁺ ion, and dissolved oxygen. In the presence of a bacterial luciferase catalyst, the light-inducing reagents would include FMNH₂, a long chain aldehyde, and dissolved oxygen. (See reaction equations (4), (5), and (6) 30 previously mentioned.)

The assay can be carried out in any suitable container having a transparent wall through which the light emissions can be detected. The assay is preferably conducted at room temperature for convenience. 35 In general, the concentrations of the various reagents in the reagent solution will be about 10⁻⁸ to 10⁻⁶ M

for antibody conjugates; 10^{-3} M for luminol when used; 5×10^{-3} M for hydrogen peroxide where used; 10^{-3} to 10^{-2} M for aldehydes where used; 10^{-2} to 10^{-1} M for pyrophosphate where used; and 10^{-2} to 10^{-1} M phosphate where applicable.

One example of a reagent solution for assaying a blood or urine sample could include peroxidase conjugated antibodies ($\sim 10^{-8}$ M), carboxy-phenylporphyrin conjugated antibodies ($\sim 10^{-8}$ M), luminol ($\sim 10^{-3}$ M), and hydrogen peroxide ($\sim 5 \times 10^{-3}$ M). Optionally, the reagent solution could also include glucose oxidase conjugated antibodies ($\sim 10^{-8}$ M), glucose ($\sim 10^{-3}$ M), and catalase ($\sim 10^{-6}$ M).

Another specific reagent solution would include peroxidase conjugated antibodies ($\sim 10^{-6}$ M), 3,10-dibromo-perylene conjugated antibodies ($\sim 10^{-6}$ M), isobutyraldehyde ($\sim 5 \times 10^{-2}$ M), dissolved oxygen, ethanol, sodium phosphate, and sodium pyrophosphate.

A further specific reagent solution would include firefly luciferase conjugated antibodies ($\sim 10^{-8}$ M), uroporphyrin conjugated antibodies ($\sim 10^{-7}$ g/ml.), ATP ($\sim 10^{-6}$ M), luciferin ($\sim 5 \times 10^{-5}$ M), Mg⁺² ion ($\sim 10^{-2}$ M), and dissolved oxygen.

It will be appreciated by those skilled in the art that many variations from the foregoing examples, shown for purposes of illustration, can be made without departing from the scope of this invention.

We claim:

1. A method for assaying antigens having multiple binding sites comprising:

- 5 a) introducing a sample into a reagent solution comprising an absorber/emitter conjugated antibody, a chemiluminescent catalyst conjugated antibody, and chemiluminescent reagents suitable for inducing a light response in the presence of the chemiluminescent catalyst conjugated antibody; and
- 10 b) detecting any light response emitted by the absorber/emitter conjugated antibody.

15 2. The method of Claim 1 wherein the chemiluminescent reagents comprise a chemiluminescent cofactor generator catalyst conjugated antibody.

20 3. The method of Claim 2 wherein the chemiluminescent reagents comprise a chemiluminescent cofactor scavenger catalyst.

25 4. The method of any preceding claim wherein the absorber/emitter is a fluorophore.

25 5. The method of Claim 4 wherein the fluorophore is selected from the group consisting of uroporphyrin, tetracarboxyphenylporphyrin, perylene, anthracene, 7-methyldibenzo (a,h) pyrene, 9,10-dibromoanthracene, riboflavin, 3,9-dibromoperylene, 3,10-dibromoperylene, and 3,10-dibromo-4,9-perylenedicarboxylate.

30 6. The method of any of claims 1 to 3 wherein the absorber/emitter is a phosphore.

30 7. The method of Claim 6 wherein the phosphore is selected from the group consisting of 2,3-butadione, 1-[carboxyphenyl]-1,2-pentanedione, and 1-phenyl-1,2-propanedione.

35 8. The method of any preceding claim wherein the chemiluminescent catalyst is selected from the group consisting of peroxidase, bacterial luciferase, and firefly luciferase.

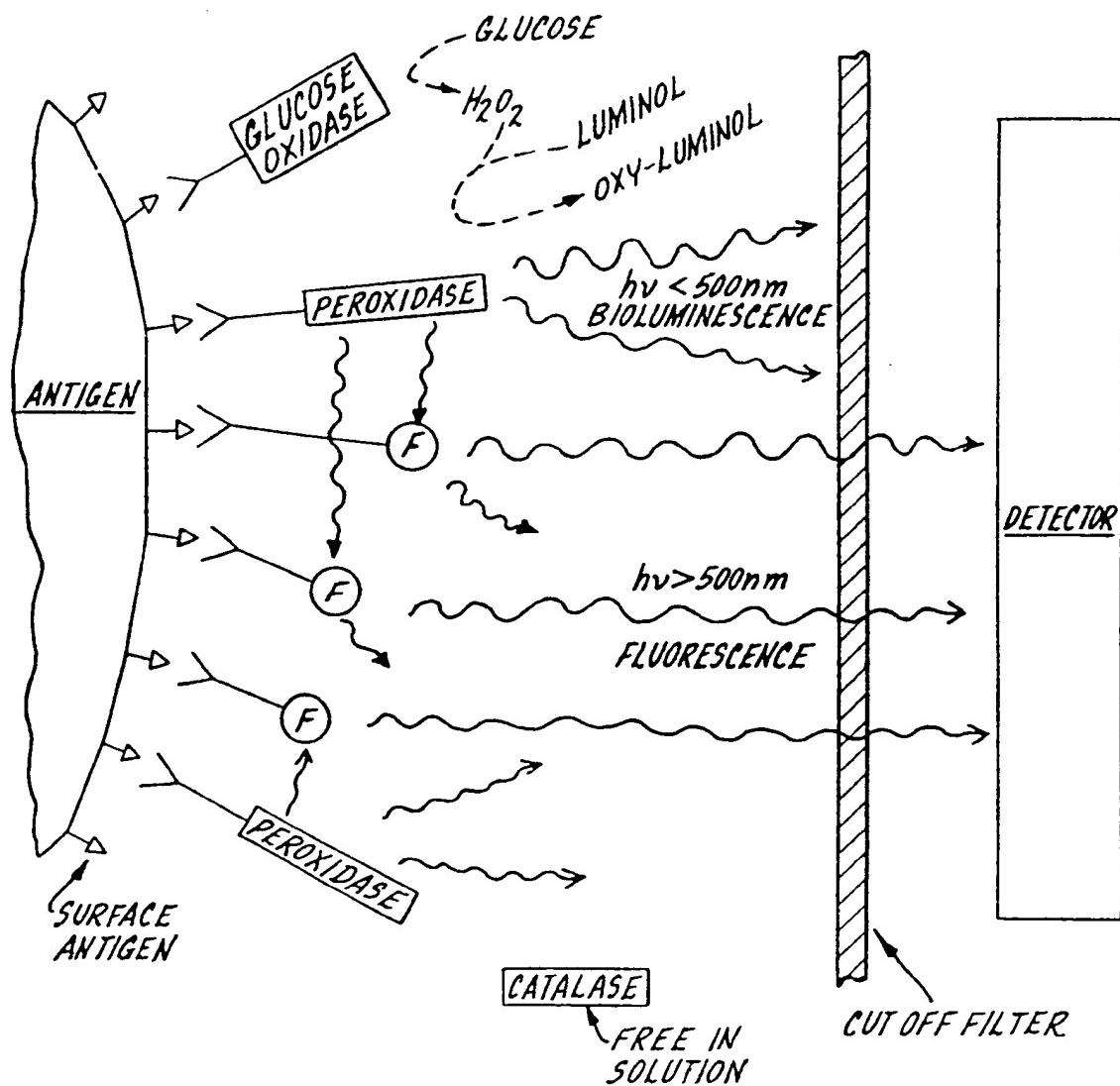
9. A reagent solution for assaying large antigens comprising an absorber/emitter conjugated antibody, a chemiluminescent catalyst conjugated antibody, and chemiluminescent reagents necessary for inducing a chemiluminescent light response in the presence of the chemiluminescent catalyst conjugated antibody.
- 5 10. The reagent solution of Claim 9 wherein the chemiluminescent reagents comprise a chemiluminescent cofactor generator catalyst conjugated antibody.
- 10 11. The reagent solution of Claim 10 wherein the chemiluminescent reagents comprise a chemiluminescent cofactor scavenger catalyst.
- 15 12. A reagent solution for assaying large antigens comprising peroxidase conjugated antibodies, uroporphyrin conjugated antibodies, luminol, and hydrogen peroxide.
13. A reagent solution for assaying large antigens comprising peroxidase conjugated antibodies, riboflavin conjugated antibodies, luminol, glucose oxidase conjugated antibodies, glucose, and catalase.
- 20 14. A reagent solution for assaying large antigens comprising peroxidase conjugated antibodies, uroporphyrin conjugated antibodies, isobutyraldehyde, dissolved oxygen, and ethanol.
- 25 15. A reagent solution for assaying large antigens comprising firefly luciferase conjugated antibodies, uroporphyrin conjugated antibodies, adenosine triphosphate, luciferin, magnesium ions, and dissolved oxygen.
- 30 16. A reagent solution for assaying small antigens comprising effective amounts of an absorber/emitter conjugated antigen, a chemiluminescent catalyst conjugated antibody, and chemiluminescent reagents necessary for inducing a chemiluminescent light response in the presence of the chemiluminescent catalyst conjugated antibody.
- 35 17. A reagent solution for assaying small antigens comprising effective amounts of a chemiluminescent catalyst conjugated antigen, an absorber/

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- 15 -

emitter conjugated antibody, and chemiluminescent reagents necessary for inducing a chemiluminescent light response in the presence of the chemiluminescent catalyst conjugated antigen.

+ 1 g.1.



REAGENTS:

Y — **F** FLUORESCENT (RIBOFLAVIN) LABELLED ANTIBODY

Y — **PEROXIDASE** PEROXIDASE CONJUGATED ANTIBODY

Y — **GLUCOSE OXIDASE** GLUCOSE OXIDASE CONJUGATED ANTIBODY

CATALASE CATALASE (NOT CONJUGATED TO ANTIBODY)

